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Characterisation of a skin secretion with adhesive properties in the ground frog *Eupsophus vertebralis* (Alsodidae)

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Abstract

Some skin secretions with adhesive properties allow frogs to distract predators and escape; their nature is poorly studied. Here, we report the sticky skin secretion released by the Patagonian frog *Eupsophus vertebralis* when stressed. This secretion contained ~ 50% proteins spanning 25–250 kDa and required a fast setting time to turn into strong adhesive, which worked well on synthetic and biological materials. Lap-shear assays with *Eupsophus* glue secretion showed average shear strength of 3.34 MPa, comparable to cyanoacrylate (5.47 MPa). These properties suggest its biotechnological value for practical applications in industrial and medical sectors.

Key Words

Amphibia, Chilean Patagonia, cutaneous glue, proteinaceous material

The genus *Eupsophus* is an endemic taxon of temperate rain forests of Chile and Argentina (Blotto et al. 2013), which includes ten species, divided into the *roseus* and *vertebralis* groups (Formas and Brieva 1992). The *roseus* group includes *E. altor*, *E. roseus*, *E. calcaratus*, *E. contulmoensis*, *E. insularis*, *E. septentrionalis*, *E. migueli* and *E. nahuelbutensis*, while the *vertebralis* group consists of *E. vertebralis* and *E. emiliopugini* (Suárez-Villota et al. 2018). During herpetological surveys on this genus, we observed that some *Eupsophus vertebralis* specimens released a sticky cutaneous secretion when they were suddenly provoked (Fig. 1A).

Gluey skin secretions have been reported in a few species, including some representatives from all three orders of extant amphibians (Evans and Brodie 1994; Tyler 2010). In general, skin secretion with adhesive properties could glue-dry leaves or debris to a predator's mouth, thus distracting the attacker and allowing the prey to escape (Evans and Brodie 1994). The distasteful and/or toxic nature of some adhesive secretions could complement the chemical defence strategy of amphibians (Phillips and Shine 2007). The most reported sticky secretion is produced by species of *Notaden*, a genus of Australian fossorial frogs, belonging to subfamily Limnodynastinae (Graham et al. 2016; Tyler 2010). When provoked by potential predators, these frogs release a sticky non-toxic material from their dorsal skin that contains mainly proteins with few carbohydrates (Graham et al. 2005; Gra-

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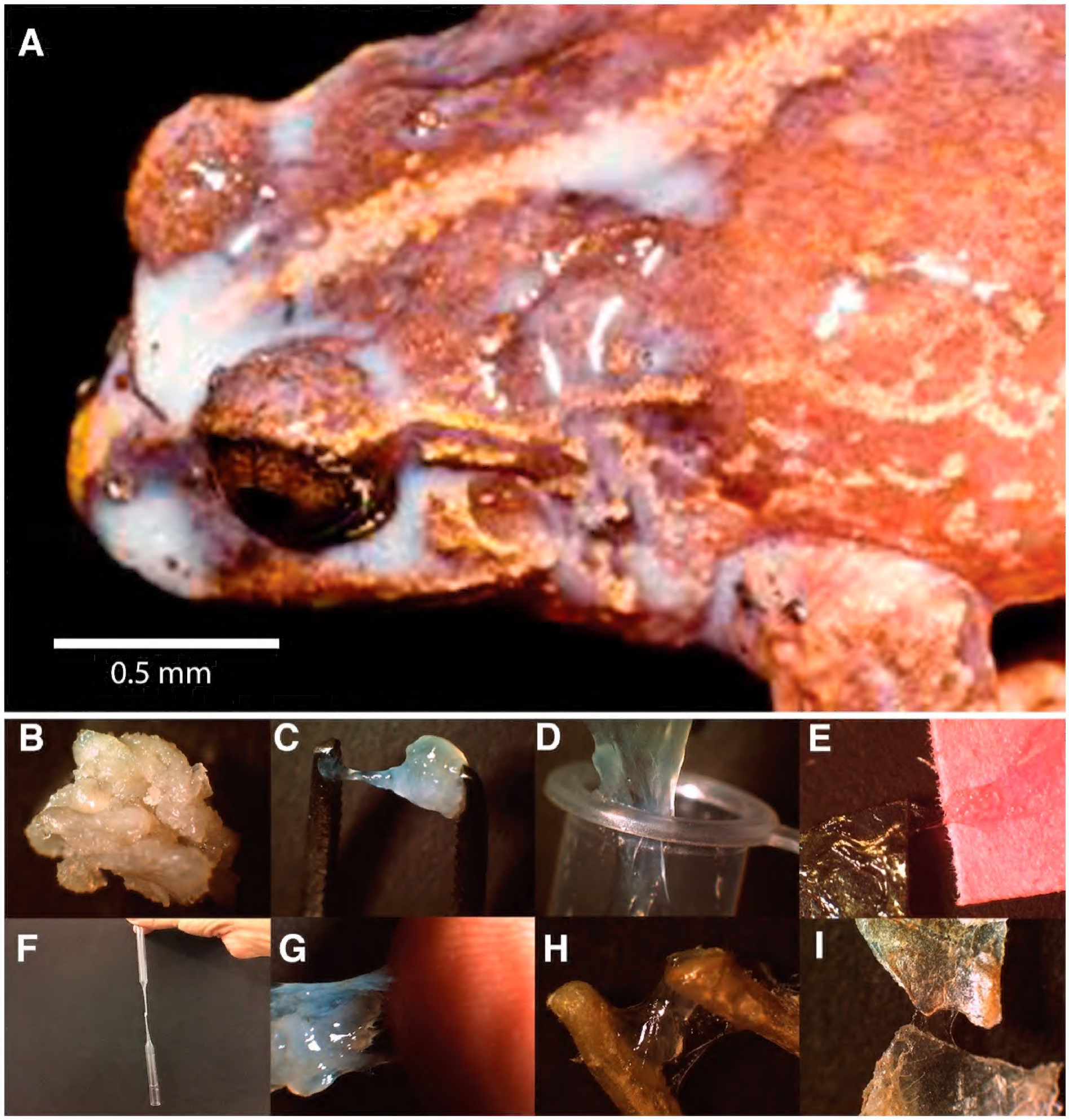

Figure 1. Adhesive properties of the *Eupsophus vertebralis* secretion. **A** Sticky secretion on *E. vertebralis*, **B** Dry secretion with silicone aspect. Secretion adhered to **C** metal, **D** plastic, **E** paper, **F** glass, **G** skin, **H** bone and **I** cartilage.

ham et al. 2016). On the other hand, sticky secretions that fasten the breeding pair during amplexus perform a role relevant to anuran reproduction. These secretions are produced from adhesive glands, which pertain to the wide and heterogeneous category of "breeding glands" (Brizzi et al. 2003). Interestingly, Siegel et al. (2008) demonstrated that adhesive glands in the sternum and forearm regions of male *Gastrophryne carolinensis* are derived from mucous glands and give negative histochemical responses to proteins.

To explore some physical-chemical properties of *Eupsophus* sticky secretions, we captured five *E. vertebralis* specimens from Llancahue, Región de Los Ríos, Chile (-39.8394, -73.1301). We carried out this procedure under the supervision and approval of the Bioethics and Biosecurity Committee of the Universidad Austral de Chile (UACh, Resolutions No. 236/2015 and 61/15) and the Servicio Agrícola y Ganadero (SAG, Resolution No. 9244/2015). Amongst these specimens, one frog released a sticky cutaneous secretion, which was collected using a sterile spatula directly from the skin and stored in ethanol (96%) at -20 °C. Subsequently, the animals were released at the collection site.

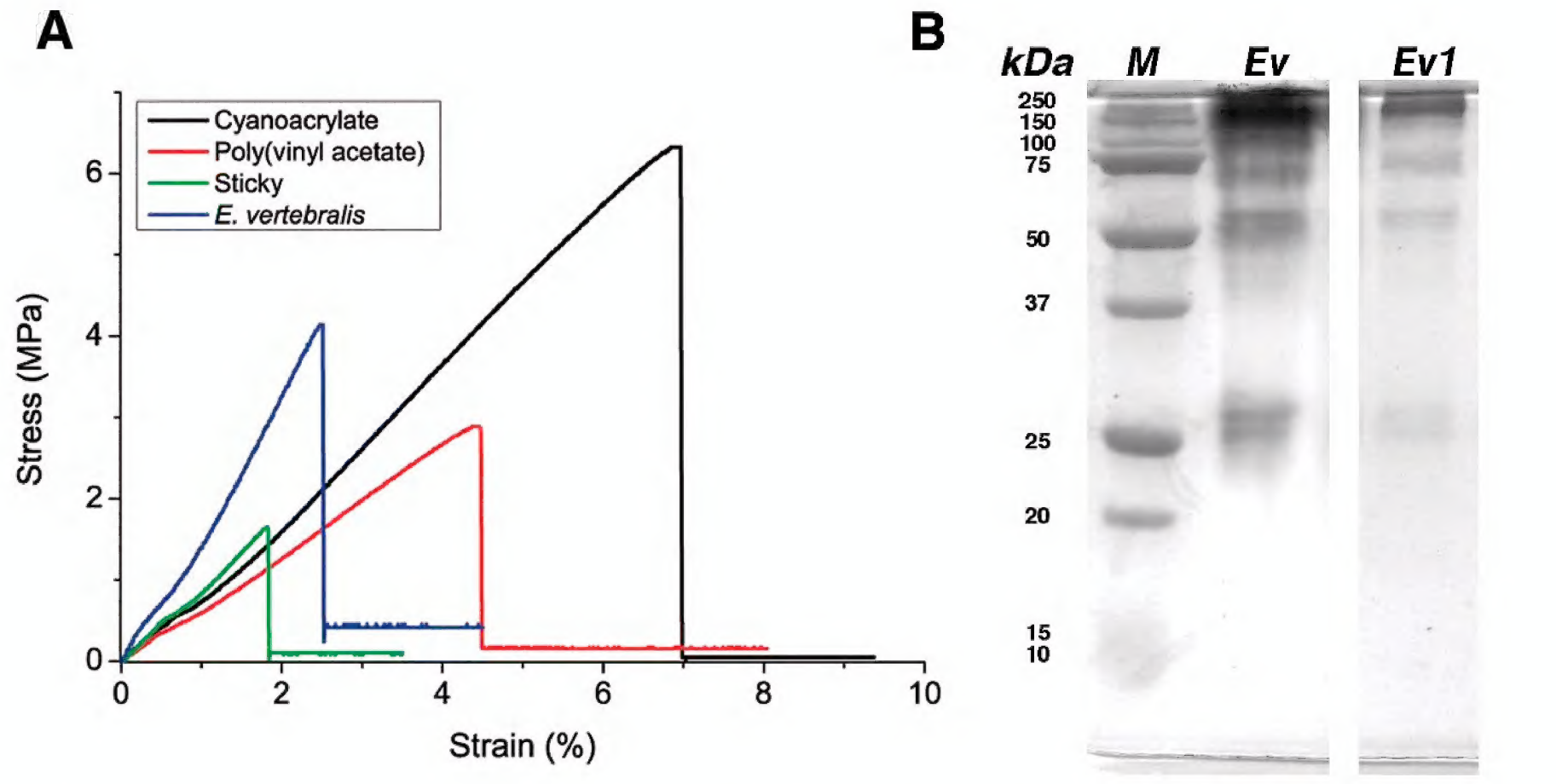

Figure 2. Physico-chemical characterization of *Eupsophus vertebralis* secretion. **A** Stress-strain plots for *E. vertebralis* secretion and three commercial glues (Pritt Stick-Fix ID:4595328, polyvinyl acetate and cyanoacrylate). See details in lap-shear tests assay in the text. **B** SDS-PAGE of *Eupsophus vertebralis* secretion samples. Each sample lane represents material from *E. vertebralis* diluted in acetic acid 5% v/v at 10 mg/µl (Ev) or at 1 mg/µl (Ev1). Marker lanes are indicated (M) and the molecular masses of the standard proteins (kDa) are shown.

Main physical properties

Sticky samples collected from *E. vertebralis* skin had a fast setting time, from ~ 10 s to ~ 2 min, turning into an adhesive with similar features to silicone (Fig. 1B). Transparent flexible samples in ethanol had less adhesive capability and turned into white-yellow crystals when removed from alcohol at room temperature. A major difficulty for analysing this adhesive secretion consisted of diluting such crystals in a suitable solution. However, we adopted effectively the strategy developed by Graham et al. (2005), using 5% (v/v) acetic acid, 10% (w/v) SDS, 5 M guanidinium hydrochloride (pH 5.0) and 10 mM phosphoric acid (H_3PO_4). Nevertheless, samples treated with SDS took more than 24 hr to dissolve. Interestingly, when crystals were rehydrated in water, the sticky properties of the secretory product were restored. The adhesion of these rehydrated samples worked on metal, paper, plastic and glass surfaces, retaining their properties in cold conditions (Fig. 1C–F, tested at -20 °C and room temperature). We also tested the adherence to biological samples such as skin, bone and cartilage (Fig. 1G–I) at room temperature.

Mechanical properties

Lap-shear tests of *E. vertebralis* dry secretion (rehydrated in distilled water) were performed in a microcomputer-controlled electronic universal testing machine (Model WDW-10E, MUE 10 kN, Time Group). For this purpose, pairs of poplar-wood craft sticks were lap-jointed by sandwiching a piece of rehydrated secretion between a 1 cm overlap. Ten of these test pieces were allowed to dry for two weeks and then tested in the universal machine using 1 kN static load cell and a cross-head speed of 1 mm/min, according to Graham et al. (2005). We also carried out this procedure on ten pieces for each commercial glue: (Pritt Stick-Fix ID:4595328), polyvinyl acetate and cyanoacrylate. Thus, the mean shear strength of *E. vertebralis* secretion was 3.34 MPa (SD = 0.98), showing bond strengths greater than dried Pritt Stick-Fix (1.63 MPa, SD = 0.87) and comparable to polyvinyl acetate (2.76 MPa, SD = 1.64) and cyanoacrylate (5.47 MPa, SD = 1.95) (Fig. 2A).

Biochemical traits

Bradford's assay on *Eupsophus* secretion revealed about 50% of proteins/dry weight, as estimated in samples diluted in 5% v/v acetic acid, using the commercial Bradford Protein kit (Co. Thermo Fischer, USA) and NanoDrop® ND-1000 Spectrophotometer. For electrophoretic separation, dehydrated secretion was eluted in acetic acid 5% (Graham et al. 2005). We carried out several assays using elutions from 1 mg/µl to 500 mg/µl: concentrations between 10 mg/µl and 100 mg/µl gave better results. Then a fraction of 10 µl of eluate solution (10 mg/µl or 100 mg/µl) was added to 90 µl of sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) loading protein buffer and then boiled at 95 °C for 10

min. Subsequently, 5 µl aliquots were loaded per lane in an SDS-PAGE composed of 12% running gel and 4% loading gel. Electrophoresis was run at 100–130V for 3 hr. Proteins were visualised by staining with Simple Blue Safe Stain (safe Coomassie G-250). This electrophoretic separation of the *E. vertebralis* secretion disclosed eight distinctive protein bands (Fig. 2B). The most abundant proteins had apparent molecular masses ranging from 150 to 250 kDa. The electrophoretic bands in this range had an odd turbulent appearance (Fig. 2B), which was more obvious at a higher dilution of the adhesive material (Fig. 2B, compare Ev and Ev1 lines).

Discussion

Our analyses disclosed adhesive properties and moderate protein concentration in secretions from South American *Eupsophus* species, comparable to those from Australian *Notaden* species (Graham et al. 2005; Graham et al. 2016; Tyler 2010). The extent of secretion release from specimens of *E. vertebralis* was highly variable. In fact, most of the individuals did not accomplish the "bulk discharge" described for cutaneous glands in other amphibians (Quagliata et al. 2008). Amphibian skin has greater diversity of glandular structures compared with other vertebrates, such as mucus glands, serous (granular) glands, lipid glands, mixed mucous-serous glands and specialised glands (sexually dimorphic skin glands, SDSG), representing modified mucous or serous glands (Brizzi et al. 2003). These cutaneous glands produce distinctive "bioactive" molecular classes, including proteins, biogenic amines and alkaloids, which are usually secreted by serous glands (Daly et al. 1987). Thus, proteinaceous material from *E. vertebralis* secretion may be physiologically discharged on to the body surface by contraction of the muscle sheaths (myoepithelia) enveloping serous or serous-derived specialised glands. In anurans, myoepithelium contraction is controlled by an adrenergic mechanism (Holmes et al. 1977). Therefore, the highly variable extents of secretion release amongst *Eupsophus* specimens represent graded defence responses and may depend on the perceived noxious manipulation.

A moderate concentration of proteins was found in dry *Eupsophus* secretion (50%) comparable to *Notaden* sticky secretion (55–60%; Graham et al. 2013). In other bio-adhesives from different animal sources, a diverse range of proteinaceous contents has been detected. In fact, protein concentration lower than 20% (i.e. in *Holothuria*; DeMoor et al. 2003) or higher than 78% (i.e. in *Plethodon*; von Byern et al. 2017) have been reported. Although some proteins, such as Nb-1R or Er_P1 (in *Notaden* and *Euperipatoides* secretions, respectively) are the major component of some glues and appear to be the key structural component in the adhesion, it should be stressed that similarities detected between adhesive materials from different animal sources most likely represent the outcome of convergent evolution (Graham et al. 2013). In this regard, Nb-1R and Er_P1 proteins have high MW (260–500 kDa), similar to the prevailing proteins of *Eupsophus* secretion (250 kDa, Fig. 2B) and all share oddly "turbulent" electrophoretic bands. Performing further electrophoretic and proteomic trials (from amino-acidic composition to increasing levels of protein structure), will allow us to establish whether such similarities are associated to comparable setting and adhesion mechanisms in these species.

Secretion from *E. vertebralis* showed adherence to various materials and its shear strength was comparable to commercial glues, resembling secretions with biotechnological potential collected from other amphibians (Graham et al. 2005; von Byern et al. 2017). For example, the secretion from *Notaden* frogs has elicited great interest for practical applications in the industrial and medical fields (Tyler 2010) and has been patented as a therapeutic adhesive derived from a natural source (Tyler and Ramshaw 2002). Indeed, *Notaden* frog glue has increased the strength in *ex vivo*-restored rotator cuff (Millar et al. 2009) and meniscus constructs (Szomor et al. 2008) from sheep. From an ophthalmological perspective, *Notaden* glue has proved to adhere successfully to collagen-coated perfluoropolyether lenses and debrided bovine corneas, supporting epithelial re-growth in a culture system (Graham et al. 2010). Moreover, tissues re-absorbed small pellets of glue implanted subcutaneously into mice (Graham et al. 2010). Thus, future research, like those carried out with *Notaden* frog glue, will allow us to know whether *Eupsophus* secretion could have a promising future in biomedical applications. The physical-chemical traits of this secretory product, such as its property of being preservable in solid state (dried), total recovery of the adhesion after hydration and capability of adhering to cold surfaces, represent potential advantages, suitable for the development of new biomimetic or biotechnological materials for application in the biological and industrial fields.

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